Report as of FY2011 for 2010VA142G: "Denitrifying bacterial community structure and diversity, and denitrification potential as affected by hydrologic design and soil properties in wetlands created in Chesapeake Piedmont, USA "

Publications

- Other Publications:
    - No publication available at this time.

Report Follows

Report on the progress of the project

May 23, 2012

Title of the project: *Denitrifying Bacterial Community Structure and Diversity, and Denitrification Potential as Affected by Hydrologic Design and Soil Properties in Wetlands*" (USGS Award # G10AP00139)

PI: Dr. Changwoo Ahn, Environmental Science and Policy, George Mason University

All the work proposed has been completed. Manuscripts are in progress. The attached is the first manuscript developed. The outcome of the research will also be presented soon in a conference as well early July 2012.

Changwoo Ahn, Ph.D

Denitrifying Bacterial Community Structure is Affected by Development of Soil Conditions in Created Mitigation Wetlands

Rita Peralta[1], Changwoo Ahn[1*], Mary Voytek[2] and Julie Kirshtein[2]
Department of Environmental Science and Policy, George Mason University, 4400 University Drive, Fairfax, Virginia 22030[1]; and United States Geological Survey, Water Resources Branch, 12201 Sunrise Valley Drive, Reston, Virginia 20192[2]
**cahn@gmu.edu*

We investigated the abundance and genetic heterogeneity of bacterial nitrite reductase genes (*nir*) in relation to soil structural attributes in created and natural non-tidal freshwater wetlands in Virginia. Soil attributes included soil organic matter (SOM), total organic carbon (TOC), total nitrogen (TN), pH, gravimetric soil moisture (GSM), and bulk density (D_b). A subset of soil attributes were analyzed across the sites, using euclidean cluster analysis, resulting in three soil condition (SC) groups of increasing wetland soil development (i.e., SC1<SC2<SC3 less to more developed) as measured by accumulation of SOM, TOC, TN, the increase of GSM, and the decrease of D_b. *NirK* gene copies detected ranged between 1.1×10^2 and 2.3×10^3 ng^{-1} extracted DNA and were highest in the most developed soil, SC3, and lowest in SC2, which had a significantly higher pH than the other two SC groups ($F = 3.8$, $p = 0.02$), suggesting soil pH may have impacts on the community structure. Gene fragments were amplified and products were screened by terminal restriction fragment length polymorphism (T-RFLP) analysis. Among 146 different T-RFs identified, fourteen were dominant and together

made up more than 65% of all detected fragments. While SC groups did not relate to whole *nirK* communities, soil attributes The outcome of the study suggests that the use of SC groups be useful to track the functional development of created mitigation wetlands.

Keywords: denitrifying bacterial community, wetland soil development, T-RFLP, qPCR, wetland functions

Mitigation wetlands are created and/or restored as a result of the national policy of ‘no net loss,’ which mandates the amelioration of the loss of wetland services through creation, replacement or enhancement (34). The degree to which this policy has been effective is debatable, with many studies indicating a mixed record of successful functional replacement (2, 52). Wetland soils serve as sites of important biogeochemical reactions that contribute to the myriad ecosystem services for which wetlands are recognized (e.g. nutrient cycling, water quality improvements, and pollution control). The degree to which soil composition (i.e. soil organic matter, total organic carbon and total nitrogen contents) begins to resemble natural soils (e.g. soil development) in created wetlands may influence biogeochemical processes, thus affecting the ability for created wetlands to regain lost wetland functions.

Created and restored wetlands tend to show lower levels of organic C and N, higher bulk densities and lower productivity than their natural counterparts (8,14, 20). Still, in most cases of wetland mitigation, vegetation has been used as the sole measure of mitigation success (7, 42). Relying on vegetation alone leaves out the role of soil physicochemical (e.g. soil moisture, pH, C content) and biological (e.g. bacterial communities) attributes in the functional development of wetlands. Soil organic matter (SOM) and carbon in particular are considered the main drivers of biogeochemical processes in wetlands (50). SOM provides the energy source and nutrients necessary for bacterial growth that can directly limit or enhance the development of ecological functions (43, 50). The ability of wetlands to support diverse metabolic and catabolic processes depends on the ability to support anaerobic and aerobic environments (35), which are directly affected by SOM and the resulting water holding capacity (16, 50).

Denitrification is one of the key ecological functions of natural wetlands extensively studied (23, 26, 31). It is a dissimilatory metabolic process that integrates a series of reductions to convert nitrate (NO_3^-) to dinitrogen (N_2), resulting in a loss of fixed nitrogen from the system. The initial reduction from NO_3^- to NO_2^- is facilitated by the synthesis of nitrate reductase under anaerobic conditions and with the presence of NO_3^-. Subsequently, NO_2^- can be reduced to NO, then N_2O and finally to N_2. The last three products being gases, released to the atmosphere at rates dependent on the efficiency of the denitrification processes, are important to water quality functions and climate change implication of wetlands. The coupled biogeochemical reactions are carried out by different members of the microbial community. Denitrifying bacteria play a significant role in the denitrification function of wetlands (23). It is known that denitrifiers constitute a taxonomically diverse functional guild with members belonging to all three domains, including more than 60 genera of bacteria, and they can represent up to 5 % of the total soil microbial community (19). Numerous studies have investigated the factors controlling denitrification in an attempt to better understand the process, mostly focusing on the roles of NO_3^- availability, O_2, and pH (22, 45, 48). These are the key regulators of denitrification rates at any particular instance. Recently, the assumption that the composition of the denitrifying community is of minor importance in controlling denitirification has been challenged by Cavigelli and Robertson (11), and Holtan-Hartwig et al. (29), which suggested that denitrifier communities vary in their tolerances to environmental conditions and stresses. Therefore, denitrifying bacterial communities may act as a medium through which environmental controls on denitrification are realized. However, little is known about the structure of denitrifying bacterial community composition and abundance as affected by soil properties.

With increasing age and additional plant growing seasons, the soil properties of a created wetland should mature and develop. An excellent indicator of soil development and quality is SOM content (8, 30), as it is a major source of nutrients (especially N) (40). SOM provides both organic N, the substrate of mineralization, and organic carbon, which is a required energy source of both mineralizing and heterotrophic denitrifying microbes (4, 23, 26). Wallenstein and others (46), in a literature review of environmental controls over denitrification, noted that C availability, pH, moisture and temperature are key factors in determining denitrifying community structure. Specifically, it has been suggested that increased soil organic carbon can be associated with bacterial diversity and may control the enzymatic/metabolic rates of the bacterial communities responsible for N processing (1, 16).

We studied the effects of soil development on denitrifying bacteria structure in created and natural wetlands in the Piedmont region of Virginia. Specifically we hypothesized that soil conditions (i.e., development and/or maturation) in created wetlands would be related to the abundance and genetic heterogeneity of bacterial nitrite reducers, the first constituents of the denitrification process that produce a gaseous product, thus removing N from wetlands. The understanding generated by this study will be useful in enhancing the chance of success for ‘functional’ wetland mitigation for future efforts.

MATERIALS AND METHODS

Site descriptions. Five non-tidal freshwater wetlands located in the Piedmont physiographic region of northern Virginia were chosen for this study (mean annual precipitation 109 cm, mean temperature min 7 ºC/ max 18ºC). Three of the wetlands are mitigation wetlands created by Wetland Studies and Solutions Inc. (WSSI) on old farmland with a predominantly herbaceous cover. The other two are natural wetlands and include bottomland riparian forested wetlands and open herbaceous wetlands.

All created wetlands contain at least a 0.3 m low permeability subsoil layer covered with the original topsoil from the site that was supplemented with commercially available topsoil to a depth of 0.2 m. This design creates a perched, precipitation-driven water table close to the soil surface and limits groundwater exchange in the wetland. Loudoun County Mitigation Bank (LC) is a 12.9 ha wetland and upland buffer complex, constructed in the summer of 2006 in Loudoun County, Virginia (39º1′ N, 77º36′ W). LC receives surface water runoff from an upland housing development and forested buffer, as well as minor groundwater inputs from toe-slope intercept seepage. LC consists of two wetland basins (LCs 1 and 2). LCs 1 and 2 are two contiguous sites separated by a berm and connected by a drainage channel with LC1 approximately 0.4 m higher in elevation than LC2. This design causes LC1 to drain more quickly leaving it inundated for shorter periods after precipitation than LC2, while LC2 can remain under standing water (e.g., < ~12 cm) for longer periods. Bull Run Mitigation Bank (BR) is a 20.2 ha wetland and upland buffer complex, constructed in 2002 in Prince William County, Virginia (38º51′ N, 77º32′ W). The site may receive water from Bull Run from a culvert structure that routes water via a central ditch through the wetland, as well as overbank flow from Bull Run, which sharply bends around the corner of the site. The wetland receives limited surface water runoff from wetlands and negligible groundwater. North Fork Wetlands Bank (NF) is a 50.6 ha wetland, constructed in 1999 in Prince William County, Virginia (38°49 ′ N, 77°40 ′ W). With the exception of minor contributions from toe-slope intercept seepage, the site is disconnected from the groundwater by an underlying clay liner. Study plots were located in two created hydrologic regimes: main pod area-fed by upland surface water runoff and a tributary of the North Fork of Broad Run that is controlled by an artificial dam; and vernal pool area - located in the south west quadrant of the wetland and fed solely by precipitation. All Vegetation in LC1, LC2 and BR is mostly herbaceous, interspersed with young tree saplings and shrubs in projected forested areas. NF vegetation includes diverse wetland herbs, shrubs, trees and submerged and floating vegetation supported by the varied hydrology. The tree communities are established and in some instances include communities extant at time of wetland creation.

Manassas National Battlefield Park (BP), is a 2,000 ha site with areas of natural wetland coverage located in Prince William County, Virginia (38º49′ N, 77º30′ W). Study plots were located in an area of herbaceous wetland within a matrix of forested floodplain. The site is connected to Bull Run by a culvert on its eastern end and also receives groundwater and upland surface water runoff. Vegetation is mostly herbaceous with a few mature trees interspersed throughout. Banshee Reeks Nature Preserve (BN) is a 290 ha site with areas of seep and riparian wetlands located in Loudoun County, Virginia (39º1′ N, 77º35′ W). These floodplain riparian wetlands receive water from groundwater springs, surface water runoff, and occasional overbank flooding from Goose

Creek. Vegetation is a mixture of herbaceous plants dominated with mature wet bottomland forest.

Soil sampling. Soil samples were collected on four dates: October and December 2010 and April and June 2011. A total of 16 study plots in the created wetlands (e.g., LC, BR and NF) and 4 plots in the natural wetlands (e.g., BN and BP) were selected. Each plot was 100 m^2 (e.g. 10 m x 10 m) and was divided into four (e.g. 5 x 5 m) quadrants. Within each quadrant, three soil samples were taken at the depth of 5-10 cm from the top by use of an auger (1 1/4" diameter) at random and combined in a polyethylene bag. All samples were kept in a cooler with ice packs to slow bacterial activity until further processing in the laboratory. At the laboratory, each bag was homogenized manually to mix all three samples for each quadrant. Any visible root or plant material was manually removed prior to homogenization.

Soil physicochemical analyses. Sub-samples taken for SOM, total organic carbon (TOC), total nitrogen (TN) and pH were air dried. Once air dried, soils were macerated using a mortar and pestle and any large constituents (e.g. rocks and large organic debris) were removed. A Perkin-Elmer 2400 Series II CHNS/O Analyzer (Perkin-Elmer Corporation, Norwalk, CT, USA) was used to analyze percent TOC (~TC) and percent TN. Sub-samples (2-3 grams of air dried soil) were separated for SOM, loss on ignition (LOI) method, and oven dried at 105 ◦C for 24 hours, weighed and placed in 405 ◦C for 16 hours. SOM was calculated as the difference between the dry soil mass and the mass of the soil after oxidation of organic matter [(dry mass – ovened at 405 ◦C mass)/(dry mass) x 100] (5). For gravimetric soil moisture (GSM), field-wet mass was measured and samples dried at 105 ◦C for 48 hours. GSM was calculated by: [(wet mass – dry mass)/(dry mass) x 100] (41). For pH determination, 10 g air dried soil samples were combined with 10 mL of deionized water, swirled and left to stabilize for 10 minutes prior to measurement (44). Bulk density (D_b) was measured once during the study period in November 2010. D_b was determined by collecting 5 cm by 10.2 cm cores, weighing the entire field-moist core, converting to dry weight based on GSM percentage, and dividing by the total volume of the soil in the core (200.2 cm^3)

Microbial Community Analyses

Extraction of DNA. DNA was extracted from approximately 0.5-1 g of soil per sample using the UltraClean® Soil DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA, USA) and following manufacturer's instructions. Extractions were quantified using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Willmington, DE, USA).

PCR amplification of *nir* fragments. Bacterial *nirK* gene fragments were amplified using primer pairs F1aCu - R3Cu (approximately 470 bp) developed by Hallin and Lindgren (24). The forward primers (F1aCu) were 5'-end FAM labeled (Operon Inc.). PCR amplification was done with 50-uL reaction mixtures in 0.5 mL Eppendorf tubes. Each reaction contained 1 uL of extracted DNA, 1.25 U of GoTaq® polymerase (Promega, Madison, WI, USA), manufacturer's reaction buffer containing 25 mM MgCl2, 2.5 mM of each deoxynucleotide triphosphate, 1.2 ug/uL non-acetylated BSA, and 20 uM of each primer. The PCR was run in a Mastercycler® gradient cycler (Eppendorf, Hamburg, Germany) with an initial denaturing step of 4 min at 94C; 35 cycles of denaturation at 94C for 30 s, primer annealing at 59C for 1 min, and extension at 72C for

1 min; then a final extension at 72C for 7 min. Products were confirmed by electrophoreses of 5uL of each reaction on 1% agarose gel.

Amplification of *nirS* gene fragments was attempted by PCR using primer pairs F1acd-R4cd (24), and also primer pairs nirS11f-nirS6R developed by Braker et al. (6). These amplifications yielded strong products for the positive control (*Pseudomonas stutzeri*), but faint if any amplification products in environmental samples. Since *nirS* gene abundances were below our detection limit, we focused on *nirK* gene amplification products for the study.

Terminal Restriction Fragment Length Polymorphism Analysis. Screening of the nitrite reducing bacterial communities was done by terminal restriction fragment length polymorphism analysis (TRFLP). Amplified *nirK* fragments were digested with *HaeIII* (New England BioLabs, Beverly, Mass., USA) restriction endonuclease enzyme for at least 4 hours at 37C. Aliquots (2-4 uL) of each digest were mixed with 12 uL deionized formamide and 0.5 uL of GeneScan-ROX500 (Applied Biosystems Instruments, Foster City, CA, USA) size standard. Mixtures were denatured for 3-5 min at 93C in and snap cooled on ice for 2 minutes. Fragment lengths were determined by using an automated DNA sequencer, model ABI 310 (Applied Biosystems Instruments, Foster City, CA, USA). The fluorescently labeled fragments were detected and analyzed by the GeneMapper® v4.1 (Applied Biosystems Instruments, Foster City, CA, USA) software. Terminal restriction fragment (T-RF) peaks from all samples were aligned using the interactive binner script (38) for R statistical software environment. T-RFs were only considered if sized between 50-400 bp with relative abundances greater than 1%. Most samples yielded detectable amounts of *nirK* gene fragments, except for samples collected in June 2010 for LC1 and BP.

Quantification of nirK gene copies. Quantitative PCR (qPCR) assays were used to quantify the abundance of *nirK* gene copies within soil bacterial communities. QuantiTect® SYBR® green PCR kits were used in 25 uL reactions containg 0.6uM of each primer, quantitect SYBR green PCR master mix, and 1 uL of DNA template in a Stratagene MX3000P thermal cycler (Agilent Technologies, La Jolla, CA, USA). Run conditions included an initial denaturing step of 15 min at 95C; 45 cycles of denaturation at 95C for 30 s, primer annealing at 55C for 30 s, and extension at 72C for 1 min; then a final cycle of 95C for 30 s, 55C for 30 s and 95C for 30 s. Standard curves were obtained with serial plasmid dilutions of a known amount of plasmid DNA containing a fragment of the *nirK* gene. Inhibitory effects of coextracted substances were tested by spiking one of three replicates with the 1e2 plasmid serial dilution to confirm amplification of correct abundances.

Data analyses. Soil condition (SC) groups were determined by cluster analysis at 70% similarity of soil physicochemical parameters that included pH, GSM, Db, TOC and TN. Statistical significance of the SC groups was verified by applying a similarity profile test (SIMPROF) which performs permutation tests at each node of the cluster analysis dendogram. SIMPROF thus determines whether each cluster set has significant evidence of a multivariate pattern different from the rest (13). We compared physicochemical and nitrite reducer community assemblages using multivariate analysis of similarities (ANOSIM) (1, 13). Additionally, principal component analysis (PCA) was used to visualize 'best fit' of plots along soil physicochemical properties and temperature gradients. All test described thus far were performed using PRIMER 6, version 6.1.5 (Primer-E Ltd., Plymouth, United Kingdom). PCA-generated principal coordinates were used for further analysis in bivariate regressions. Shannon–Weiner's diversity index (H') was calculated based on the observed fragments generated by T-RFLPs of the wetland soils. Where H' is equal to ∑[pi (ln pi)] and pi is the peak area (i.e., relative abundance of a particular T-RF) in the ith observed taxonomic unit (OTU) (e.g. base pair length at which T-RF is detected).

Analysis of variance (ANOVA) was used to compare soil physicochemical variables, T-RF diversity and *nirK* gene copies abundance between soil condition groups. Dunnett's posthoc tests for uneven variances were carried out for each ANOVA to determine between-group differences. Bivariate regressions of soil properties and *nirK* abundances were performed to confirm significant relationships between factors. ANOVAs and regressions were conducted using SYSTAT 12 (Cranes Software International Ltd).

Redundancy analysis was performed on denitrifying bacteria community composition based on *nirK* gene T-RFLP. Soil physico-chemical attributes used for redundancy analysis (RDA) included pH, SOM, TOC, TN, GSM and temperature (C). RDAs were carried out using CANOCO, version 4.5. (Biometrics-Plant Research International, Wageningen, Netherlands). The significance of the relationships between the soil physicochemical variables and the T-RFs were calculated by use of Monte Carlo permutations and $p<0.05$ were considered non random.

RESULTS AND DISCUSSION

Development of soil properties. Soil properties were found not to be solely defined by the age of the wetland or even necessarily homogeneous within each site (Table 1). SOM and TN were significantly higher in the forested natural wetland (BN), the oldest created wetland (NF) and one of the youngest created wetlands (LC1) with SOM contents of 4.1% up to 5.6% and TN from 0.24% to 0.48%. The same trend was found for TOC and GSM content, with the exception of NF which showed a non-significant higher content than BR, BP and LC1. Soil pH values ranged from 4.2 to 6.5 with NF containing the highest and BP the lowest values.

Soil properties develop through the accumulation of SOM which is closely associated to age related factors such as seasonal plant senescence (2). However, age based soil development trajectories have been found to be highly variable and not predictive of plant community development (18). SOM accumulation can vary due to variables that may facilitate or impede autochthonous (e.g. seasonal plant senescence) and allochthonous or allogenic (e.g. sediment brought by flooding or runoff) sources of organic matter. The construction process itself can compact soils increasing Db and decreasing microtopography leading to a loss in water holding capacity and loss of SOM (33). Therefore comparison of soil development within and between wetland sites may be better achieved by identifying soil attributes that contribute to soil development. Accumulation of SOM, TOC, and TN along with the resulting lower Db and increased GSM have been identified as structural attributes correlated with increased plant (18) and biogeochemical productivity (50).

We compared plots from four created and two natural wetlands by grouping them along a soil condition gradient. SC groups were identified by cluster analysis of all plots discriminated by five easily measured soil physicochemical properties; pH, GSM, Db, TOC and TN. SC groups effectively discriminated plots according to progressive soil development/maturation (e.g., SC1<SC2<SC3), irrespective of site (Table 1). SOM ranged on average from 3.2% in SC1 to 5.0% in SC3 and was significantly different for each group ($p<0.01$). While TOC and TN are closely related to SOM, there was no difference in TOC and TN contents between SC1 and SC2 ($p>0.05$). TOC and TN were highest in SC3 ($p<0.05$) which on average had contents of 2.0% and 0.2% respectively (Table 1). Db and GSM followed a similar pattern indicating that SC1 was the least mature group having higher Db ($p<0.01$) and lower GSM ($p<0.01$; Table 1). These values indicate mineral soils and are comparable to young (e.g., <20 years old) created wetlands in Virginia (TOC 0.3-4.0%) (33), North Carolina (SOM 0.6-4.03, Db 0.99-1.64 g^{-1}cm^{3}) (8), and New York (SOM 6.2%, Db 1.1 g^{-1}cm^{3}) (2). Natural wetlands were also included in the SC groups and while SOM and TOC contents are lower than reported in Pennsylvania (SOM mean 11.5%) (10) and Maryland (TOC mean 5.7%) (28) they were comparable to other Virginia natural sites (0.7-7.7%) (33).

Seasonal variability was observed in SOM with October and December having a significantly higher content than April and June (Table 2). TOC followed a similar trend with the exception of October not being significantly higher than April and June. TN contents were highest in June and lowest in April with no significant difference between October and December (Table 1). GSM measurements ranged from 29% to 40% with December and April significantly wetter than October and June (Table 2). However, all the differences observed in the variables seemed within the ranges of natural variation, based on several studies conducted on the same wetlands as investigated in this study (see 1, 18, 33, 50). Temperatures ranged on average from 1.3 C in December to 20.5 C in June.

Abundance of nitrite reducers among SC groups. Denitrifying bacterial community abundances were assessed by quantifying the number of *nirK* functional gene copies per sample. *NirK* gene copies ranged between 3.6 x 10^4 and 3.4 x 10^7 copies g^{-1} soil. To account for any differences in biomass between samples, we also calculated the abundance of copies normalized to the amount of extracted DNA. The gene copy numbers ranged from 1.2 x 10^2 to 4.4 x 10^3 copies ng^{-1} extracted DNA. It is difficult to find comparable studies in wetland soils quantifying *nirK* genes, however our gene copy abundances were lower than those published for a paddy field in Taoyuan, China (2.0 x 10^8 to 2.4 x 10^8 copies g^{-1}) (12) and riparian soils in Thomas Brook watershed, Canada (2.2 x 10^9 copies g^{-1}) (15). Other more comparable values were found in studies looking at *nir*K gene copy abundances in various soils (9.7×10^4 to 3.9×10^6 copies g^{-1}) (25) and organic humus in south Bohemia (2.7 x 10^4 to 1.2 x 10^6 copies g^{-1}; 3).

Abundances of *nir*K gene copies, whether calculated per gram soil or per ng of DNA, were greater in the plots with greater SOM, TOC, TC, GSM and lower D_b when comparing soils of similar pH (Table 1). SC3 plots had the highest abundance ($p=0.02$) followed by SC1. Denitrification is a facultative process that requires anaerobic conditions, such as those observed in inundated soils. The more developed SC plots have higher levels of SOM which not only provide an energy source but also can contribute to lower Db. The lower Db in turn increases pore space that allows for greater water retention and may lead to higher GSM. The resulting soil matrix is better able to maintain anoxic conditions, that may be able to give an advantage to those bacteria that are able to use an alternate (i.e. NO^-_2) terminal electron acceptor than O_2.

When pH was considered, the lowest *nirK* gene abundances occurred in soils with higher pH; SC2 contained an order of magnitude less gene copies than either SC1 or SC3 (Table 1). SC2 SOM and GSM contents were higher on average than SC1, yet this group contained the lowest number of *nirK* gene copies (Table 1 ; $p<0.01$). However, the relationship between higher pH and low copy numbers was not limited to SC2 plots, a bivariate regression of pH and *nirK* abundances supports that our soils have a negative relationship with increased pH ($R=0.27, p=0.03$). pH has been extensively researched as an environmental factor affecting bacterial communities (21, 32). Among the few studies that have specifically linked *nirK* communities to pH, Barta and others (2010) found that *nirK* gene abundances had a positive relationship with pH. Furthermore, they found that in Bohemian Forest soils the lower threshold was a pH of 5. Our data show that this relationship may not apply consistently observed to all soil types and may point to adaptations to the lower pH by soil denitrifying communities (46). In fact, the effect of pH is not necessarily a direct one on the denitrification processes, and may be more related to other environmental and biological factors (39). For instance, the availability of organic carbon and other nutrients can be diminished in acidic environments, leading to lower energy source and reducing the activity of the heterotrophic microbial community as a whole and the denitrifying community in particular (39). Finally, we have to consider a significant limitation of this study, where we focus on bacterial nitrite reducers, which constitute one group that perform one intermediary step in the whole denitrification processes. Studies have linked lower pH levels to a higher ratio of N2O:

N2 when measuring denitrification yields (9, 17). Therefore, the low abundance of *nirK* genes in NF may indicate a higher community composition of denitrifiers that convert N2O to N2.

To further link the relationship between nitrite reducers and soil condition groups we looked at the PCA of physicochemical attributes (Fig. 1) which clearly demonstrated SC groupings. Forty seven percent of all the variation between plots was explained by PC1, which was negatively related with SOM, TOC, TN and GSM (Fig. 1). PC1 was also negatively correlated with *nirK* abundances (*adjusted* $R=0.318$, $p<0.01$), indicating that as SOM, TOC, TN and GSM values increased so did the nitrite reducing community. PC2 was negatively correlated with pH and temperature and was not significantly correlated to *nirK* gene abundances (*adjusted* $R=0$, $p=0.63$). The effect of temperature can be appreciated by looking at changes in the abundances of the *nirK* gene copies over the four different sampling periods (Table 2). April has the lowest abundances ($p<0.01$), but by June these are higher yet still not significantly different from those in October and December. A cyclical pattern may exist where growth is maximized at the end of the growing season, after a period of higher temperatures. That is, there could be a time lag in response of *nirK* bearing community to temperature changes, whereby the effect of higher temperature causing increased abundance is not observed until June (rather than in April), and similarly, the effect of lower temperature causing decreased abundance is not observed until December (rather than October) and lowest in April (Table 2). Since gene copy numbers do not measure activity and denitrification is a facultative process, the abundance of nitrite reducers is not in and of itself indicative of functional development. However, the abundance of a functional gene (e.g. *nir*) in a community may be used as a baseline for comparing potentials for functional development. Relating the abundances with SC attributes further can indicate a link between the soil structural controls and their effect on functional development. Nitrogen flux rates for example can increase with soil structural measures equivalent to SC (50). The increased nutrient availability and energy source in soils with higher SOM content, along with moisture retention that enable lower redox potentials, do affect potential denitrification rates (50). Our results show that nitrite reducers are responsive to soil development, (e.g. increased SOM, TOC, TN and GSM and lower Db) and their abundance may serve as good surrogates for functional development in terms of N cycling in created wetlands.

Denitrifying community diversity and structure among SC groups. The denitrifying bacterial communities from plots classified by one of the three SC groups were evaluated by T-RFLP analysis of the amplified *nir*K gene fragments. We detected a total of 146 different T-RFs in all of the samples. The average numbers of fragments observed in each group were 131, 99 and 110 for SC1, SC2 and SC3, respectively. Nitrite reducer community structures were not explained by SC groupings (ANOSIM *Global R=0.046, p=0.1*). In contrast, differences were detected by sampling period (ANOSIM *Global R=0.23, p=0.01).* Eighty-seven T-RFs were detected in October, 93 in December, 129 in April and 56 in June. Of these only 34 T-RFs were detected in all sampling periods. The greatest differences were between April and June (*ANOSIM R=0.39, p=0.01*). Comparable temporal variations in *nirK* community structures have been identified in arable soils (51) and a wetland mitigation bank in Illinois (36). The temporal variability indicates greater sensitivity of the *nirK* communities to seasonal factors such as soil temperature, soil moisture and nutrient inputs that may vary seasonally.

Diversity values ranged from 2.7 to 3.2, no significant difference was found by SC (Table 1). These values are lower than a comparable study using T-RFLPs to assess *nirK* gene diversity in marsh (H' = 3.6) and upland (H' = 4.4) soils in Michigan (37). Admittedly the comparison is somewhat limited due to the range of primers and methods used to study denitrifying bacteria communities. While no significant differences were found between SC groups, the average values were higher in SC3 than the other two groups (Table 1), indicating mature soil structure might support higher diversity of denitrifying bacterial community. Temporal variations in diversity were observed in April and June, which both the highest and lowest H' values, respectively (Table 2). The lower diversity in June was also affected by the exclusion of LC1 and BP as these soils did not produce PCR products (e.g. *nirK* gene fragments). April diversity values were affected by an increased number of fragments that had very low relative abundances (data not shown). H' is considered a useful diversity index that takes into account richness and evenness; however it is known to be more sensitive to the variability in abundance of rare groups (27). Therefore, although the April diversity values are significantly higher than other sampling periods, we have to be cautious of the extent to which the rare groups may contribute to overall structure.

Considering the effect of rarer species on diversity values we identified fourteen T-RFs, from the 146 detected, which together made up more than 65% of all detected T-RFs. Dominant T-RFs did correspond to four main factors; 1) pH, 2) TN, 3) temperature and 4) GSM, SOM and TOC (Fig. 2). Specifically we found three fragments (e.g., T-RFs-311, 277 and 141) were positively correlated with TN and negatively with pH. While two (e.g., T-RFs 281 and 313) increased in relative abundance with decreasing pH and lower TN. Four fragments (e.g., T-RFs 131, 173, 243 and 337) increased in abundance with increased GSM, SOM and TOC. Four others (e.g.,T-RFs 135, 141, 209 and 275) were positively correlated with temperature (Fig. 2). The importance of dominant T-RFs in *nirK* soil communities was explored by Wertz and others (2009) in Canadian agricultural soils. The study compared whole and active (using mRNA transcripts from entire community) *nirK* community composition, concluding that the active portion was relatively stable and more abundant in all sampled soils (49). This kind of variations in *nirK* community structures have also been observed in stream sediments along an urbanization gradient (47). Another study revealed *nir*K (and *nos*Z) community shifts in stream sediments along a disturbance gradient created by urbanization (e.g. percent impervious cover and water quality indicators), as measured by TRFLP community analysis (47). This can be seen as somewhat analogous to results here. Some of the same physicochemical constituents important to the dominant TRFs in our study, and which may be different in constructed versus natural wetlands, and differing soil conditions, such as TN, TOC, and pH, are also those that changed with urbanization. All of these studies show that *nirK* bearing denitrifiers respond to physicochemical changes in the environment, making *nirK* community structure assessment a useful tool.

Relative abundance shifts of the dominant T-RFs were apparent by SC groups and sampling period (Fig. 3). Two dominant fragments (e.g., T-RFs, 277 and 275) had higher relative abundances in SC1, while two others (e.g., T-RFs 209 and 173) had lowest abundances in this group. SC2 plots collectively had lower abundance of four dominant fragments (e.g., T-RFs 135, 275, 279 and 281) and highest of fragment 119. SC3 had the most evenly distributed abundance profile and had the highest abundance of T-RF-135

(Fig. 3). Sampling period also played somewhat a role in dominant T-RFs composition. Two, 119 and 281 bp, were relatively higher in April. Eight dominant T-RFs (131, 135, 173, 209, 243, 275, 313 and 337) had higher relative abundances in October and December (Fig. 3). These results highlight the relationship between soil development and the structural characteristics of a biological component of the denitrification process. Denitrifier communities in the wetlands studied seem to be dominated by a few members that respond to soil physicochemical attributes.

CONCLUSIONS

We investigated a subset of soil denitrifying communities (e.g., nitrite reducers) and soil properties in created and natural non-tidal freshwater wetland soils in Virginia. Soil condition groups classified study plots according to increased soil development using five easily measured physicochemical attributes; TOC, TN, Db, pH and GSM. SC groups in turn had significant relationships with nitrite reducers in terms of abundance with its greater abundance associated with mature soil properties. While SC groups did not relate to whole *nirK* communities, soil attributes that identified SC groups did significantly correlate to dominant members of the community. Furthermore, this study highlights the need for further study of the relationship between the different constituents of denitrification and soil pH. The outcome of the study suggests that soil properties and their maturation are to be used in post-construction monitoring to better assess functional development of created mitigation wetlands.

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